

Measurement of Antiviral Activities Using Recombinant Human Cytomegalovirus

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(Received September 14, 2000 / Accepted November 25, 2000)

For rapid and sensitive measurement of antiviral activities, application of a recombinant virus containing firefly luciferase gene was attempted. Recombinant human cytomegalovirus (HCMV) containing luciferase gene driven by HCMV late gene pp28 promoter (HCMV/pp28-luc) was used to test the antiviral activities of three known compounds and the result was compared with results from the conventional plaque assay for measuring the production of infectious viruses. When human fibroblast cells were infected with HCMV/pp28-luc, luciferase activity was observed at 2 days after infection and reached maximum at 6 days after infection, whereas the production of infectious virus was maximal at 4 days after infection. The antiviral activities of ganciclovir, acyclovir, and papaverine were measured in HFF cells infected with HCMV/pp28-luc and the luciferase activity was compared with the infectious virus titers. Luciferase activity decreased as the concentration of ganciclovir or papaverine increased, while there was a slight decrease in luciferase activity with acyclovir. The level of the decrease in luciferase activity was comparable to the level of decrease in the production of infectious virus. Therefore, the antiviral assay using recombinant virus HCMV/pp28-luc resulted in sensitivity similar to the conventional plaque assay with a significant reduction in assay time.

Key words : human cytomegalovirus, luciferase, antiviral activity

Human cytomegalovirus (HCMV) is a ubiquitous human pathogen, causing a wide range of clinical or subclinical syndromes. People may be infected with HCMV early in life, usually without significant clinical symptoms. After primary infection, like other members of the herpesvirus group, HCMV remains in the human body and establishes a latent infection. Later in life, with natural or acquired immune dysfunction such as organ transplantation or acquired immunodeficiency syndrome (AIDS), reactivation of HCMV may occur and this often causes severe clinical consequences with high morbidity and mortality (7). When a fetus is infected, HCMV may cause severe, generalized symptoms of cytomegalic inclusion disease (CID) of the neonate, which results in complex developmental and neurologic abnormalities.

Currently, clinically available drugs for antiviral therapies against HCMV infection include ganciclovir (GCV), foscarnet, and cidofovir. All these drugs have problems of low oral bioavailability, toxicity and the occurrence of resistant mutants (13). Thus novel anti-HCMV compounds with improved efficacy and lower side effects are needed. Development of novel antivirals, however, is

tedious and time-consuming. The development and characterization of antivirals usually depend on appropriate screening assay methods. Plaque assay is the most frequently and widely used method for measurement of antiviral activities since it measures actual production of infectious viruses (9, 18). Modification of plaque assay to get rapid results has been reported (22). But plaque assay has the critical disadvantage of being labor-intensive and time-consuming and the results may vary depending on the virus strains and cells used as well as varying results from different laboratories, implying a necessity for a standardized assay method (17). Therefore alternative antiviral assay methods have been developed in an attempt to reduce the assay time and to produce results comparable to the plaque assay. These include DNA-DNA hybridization (11) and application of flow cytometry (15, 20). Recently, recombinant viruses expressing green fluorescent protein have been developed and used for screening antiviral agents against pseudorabiesvirus (14) and HCMV (18).

In this study, we used a recombinant HCMV (HCMV/pp28-luc) containing firefly luciferase gene driven by HCMV late gene pp28 promoter for measuring antiviral activities of three compounds and the results were compared with results from the plaque assay. We found that use of HCMV/pp28-luc could be a rapid and sensitive

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alternative method for measuring antiviral activities of potential antiviral agents.

Materials and Methods

Cells and virus

Primary human foreskin fibroblast (HFF) cells were used in this study. Cells were propagated in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) under a 5% CO₂ atmosphere at 37°C. The recombinant HCMV (HCMV/pp28-luc) containing firefly luciferase gene under control of HCMV late pp28 gene promoter was obtained from Dr. Gary Hayward (Johns Hopkins University Medical School, Baltimore, MD, USA). HCMV/pp28-luc was developed initially to exploit sensitive assay for HCMV immediate early (IE) gene function that could be carried out in virus-infected cells (1). The DNA fragment containing pp28 promoter-luciferase gene was inserted in an extragenic location between US9 and US10. To prepare virus stocks, HFF cells were infected with HCMV at a multiplicity of infection (MOI) of approximately 0.01 plaque forming units (PFU) per cell. The medium was changed 4 to 5 days postinfection and the virus was harvested at 8 to 10 days after infection by two cycles of freezing and thawing. The cell extract was sonicated and stored at -70°C

Antiviral compounds

A well-known anti-HCMV drug ganciclovir (GCV) was obtained from Syntex (Mountain View, CA, U.S.A.). Acyclovir (ACV) was purchased from Burroughs Wellcome (Research Triangle Park, NC, U.S.A.). GCV and ACV were dissolved in water. Papaverine was in the liquid form for injection (30 mg/ml) and purchased from Eli Lilly (Indianapolis, IN, U.S.A.).

Plaque assay

To determine the titer of infectious virus, a serially diluted (10-fold) virus sample was inoculated (0.2 ml per 35 mm culture dish) onto a confluent monolayer of HFF cells and incubated for 1 hr with gentle rocking every 15 min. This was followed by a wash with phosphate buffered saline (PBS) and the cells were overlaid with a semi-solid medium consisting of DMEM with 2% FBS, 0.25% agarose (Type II, medium EEO, Sigma Chemical Co., St. Louis, MO, USA), 100 units/ml penicillin, 100 µg/ml streptomycin, and 1 µg/ml Fungizone (Flow Lab., McLean, VA, USA). After 7 days, a second overlay was added. Cells were fixed with 10% formalin in 0.85% saline about 2 weeks after virus infection. The cell monolayer was then stained with 0.03% methylene blue and the number of formed plaques was counted.

Luciferase assay

Cells grown in 35 mm tissue culture dishes were infected

with HCMV/pp28-luc at MOI of 1 PFU/cell. After 1 hr adsorption at 37°C, the virus inoculum was removed and the cells were fed with fresh medium containing 2% FBS. On certain days after virus infection, cells were collected by centrifugation, resuspended with 0.25 M Tris (pH 7.9)-1 mM dithiothreitol and subjected to three cycles of freezing in an ethanol bath at -70°C and thawing in a water bath at 37°C for 5 min each. The extract was clarified by centrifugation at 12,000 rpm for 10 min at 4°C. Fifty ml of supernatant was combined with 150 µl of reaction buffer (25 mM glycylglycine, pH7.8, 15 mM MgSO₄, 5 mM ATP, and 4 mM EGTA) and added to the wells of 96 well plates. Luciferin (0.25 mM, Sigma Chemical Co.) was mixed and the resulting luminescence was measured in relative light units (RLU) using a luminometer (Microumat LB96P, Berthold, Germany).

Results and Discussion

Expression of luciferase in HFF cells infected with HCMV/pp28-luc

Firefly luciferase gene has been widely used for reporter gene assay together with bacterial β-galactosidase (β-gal) and chloramphenicol acetyltransferase (CAT) genes to characterize the regulation and function of eukaryotic genes (2, 5). Luciferase assay has advantages over the other two assay systems in that it is rapid and not affected by the sample prepared. Most of all, luciferase assay is very sensitive and can detect as little as 1 fg to 20 ng of luciferase in the sample (5, 8, 12). Therefore luciferase assay has been used when the sensitivity is the prime concern. In this study, recombinant HCMV containing luciferase genes under the control of HCMV late pp28 gene promoter was used and the level of luciferase gene expression was compared with the level of production of infectious virus. The reason for choosing HCMV late gene pp28 was that the purpose of this study was to compare plaque assay and luciferase assay in quantifying infectious virus as a result of virus replication in the cell in the presence of potential antiviral agents. If certain antiviral agents affect immediate early or early gene expression, this may not correlate with inhibition of the production of infectious virus. Late genes such as pp28 are expressed after virus DNA replication, and the effect on late gene expression could be correlated with the effect on the production of infectious virus.

The purpose of this study is to develop a rapid and sensitive assay system using luciferase reporter gene comparable to plaque assays. Hence the earliest time for reasonably high luciferase gene expression in HFF cells infected with HCMV/pp28-luc was determined. HFF cells were infected with HCMV/pp28-luc and the cell extracts were prepared at 2 day intervals. The results of luciferase and plaque assays are shown in Fig. 1. Luciferase activity began to appear as early as 2 days after virus infection and

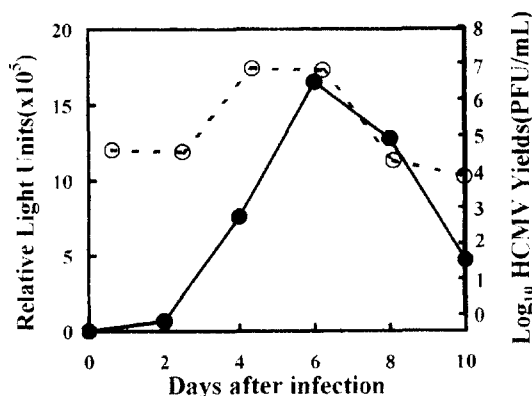


Fig. 1. One-step growth cycle of HCMV/pp28-luc. HFF cells were infected with HCMV/pp28-luc. Every 2 days the infected cells were harvested and assayed for infectious virus titers by plaque assay (○) or luciferase activity (●) as described in Materials and Methods.

reached maximum at 6 days. Thereafter luciferase activity decreased. On the other hand, infectious virus titer was at its maximum at 4 days after infection and declined thereafter. Although the luciferase activity was maximal at 6 days after virus infection, we chose the time for the luciferase assay at 4 days after infection, since virus production was the highest at 4 days and a relatively high level of luciferase activity was observed at this time. Moreover, the purpose of this study is to develop a rapid assay system for measuring antiviral activity.

Comparison of luciferase and plaque assays

Ganciclovir (GCV) is an analog of acyclovir (ACV), which was developed as an anti-herpesviral agent. ACV is preferentially phosphorylated by herpesvirus thymidine kinase (TK) to ACV monophosphate, which is then converted to ACV triphosphate by cellular TK. ACV triphosphate is used preferentially by herpesvirus DNA polymerase and incorporated into herpesvirus DNA, thus terminating viral DNA synthesis. Since alpha-herpesviruses such as herpes simplex virus and varicella-zoster virus encode viral TK, they are susceptible to the antiviral activity of ACV. HCMV does not encode its own TK, thus is not as vulnerable to antiviral action of ACV as alpha-herpesviruses. Instead, HCMV is susceptible to GCV, whose precise anti-HCMV mechanism is not well understood. GCV is phosphorylated, incorporated into viral DNA and blocks HCMV DNA replication (19).

GCV was used in this study to compare the sensitivity and fidelity of luciferase assays with the sensitivity and fidelity of plaque assays. GCV inhibited HCMV multiplication in a dose-dependent manner in the concentration range of 0.3 and 10 μ M (Fig. 2). At the same concentration range, GCV also inhibited luciferase activity of HFF cells infected with HCMV/pp28-luc in a dose-dependent manner. Therefore, higher anti-HCMV activity at higher concentrations of GCV measured by plaque

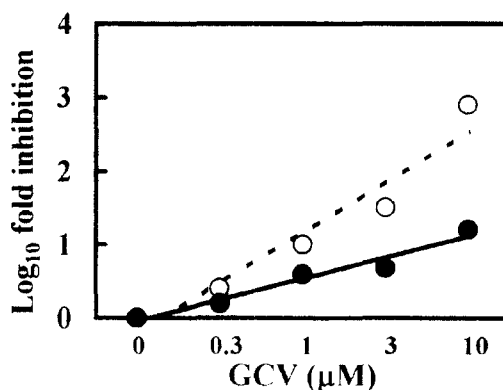


Fig. 2. Inhibition of HCMV replication by ganciclovir. HFF cells were infected with HCMV/pp28-luc, and at 4 days after infection cells were harvested and assayed for infectious virus titers by plaque assay (○) or luciferase activity (●).

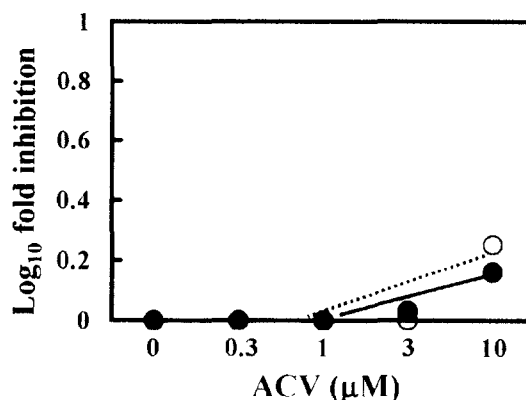


Fig. 3. Effect of acyclovir on HCMV replication. HFF cells were infected with HCMV/pp28-luc. Four days after infection, the cells were harvested and assayed for infectious virus titers by plaque assay (○) or luciferase activity (●).

assay was also observed with luciferase assays. ACV is not a potent anti-HCMV compound. As shown in Fig. 3, ACV did not significantly inhibit HCMV multiplication when measured by either plaque assay or luciferase assay, as expected.

Papaverine is a smooth muscle relaxing agent and known to inhibit HCMV multiplication (3). The mode of action of papaverine in inhibiting HCMV multiplication appears to block HCMV-induced early cellular responses including cAMP, cGMP and calcium (4, 21). Since papaverine seems to have a different mode of antiviral action from GCV, testing the antiviral activity of papaverine by luciferase assay is merited. Papaverine inhibited HCMV multiplication in a dose-dependent manner. At a concentration of 10 μ M, papaverine blocked HCMV multiplication by approximately 1,000-fold, and the antiviral activity decreased as the concentration of papaverine decreased (Fig. 4). The papaverine dose-dependent experiment with luciferase assay resulted in a very similar pattern of inhi-

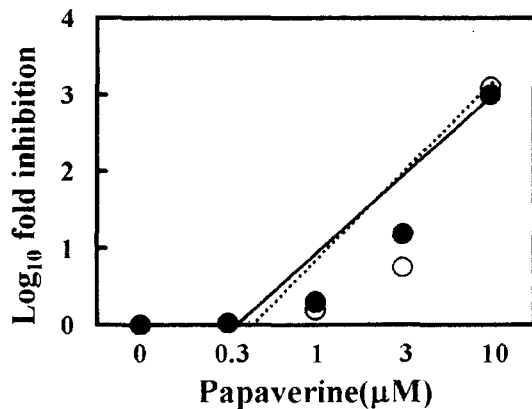


Fig. 4. Inhibition of HCMV replication by papaverine. HFF cells were infected with HCMV/pp28-luc. Four days after infection, the cells were harvested and assayed for infectious virus titers by plaque assay (○) or luciferase activity (●).

Table 1. Comparison of plaque assay and luciferase assay

Drugs	Plaque assay (PFU ^a /mL)		Luciferase assay (RLU ^b)	
	ED ₅₀ (μM)	ED ₉₀ (μM)	ED ₅₀ (μM)	ED ₉₀ (μM)
Ganciclovir	0.45	1.05	0.44	5.94
Papaverine	1.02	1.8	1.14	2.01
Acyclovir	10.4	≥30	≥30	≥30

bition of luciferase activity. In fact, the two graphs showing the dose-dependent inhibition of HCMV multiplication and luciferase (Fig. 4) are highly superimposable.

Using the data shown above, ED₅₀ (effective dose 95%) and ED₉₀ (effective dose 90%) were calculated and are summarized in Table 1. The ED₅₀s obtained from plaque assays and luciferase assays for GCV and papaverine were almost identical (0.45 μM vs 0.44 μM for GCV, and 1.02 μM vs 1.14 μM for papaverine). Although the ED₉₀ for GCV obtained with luciferase assays was higher than that obtained with plaque assays, the ED₉₀ for papaverine obtained with luciferase assays was comparable to the ED₉₀ obtained with plaque assays. Taken together, the data indicate luciferase assays can be used to evaluate the antiviral activity of certain compounds and highly comparable to well-established antiviral assay of plaque assays.

Currently several reports are available concerning the use of recombinant herpesviruses for the study of regulation of viral gene expression or antiviral assays (6, 10, 14, 16). Recently Marshall *et al* (19) reported recombinant green fluorescence protein (GFP)-expressing HCMV as a tool for screening antiviral agents. The GFP-expressing vector was inserted between HCMV US9 and US10 genes. They claimed that the GFP-based antiviral assay is rapid, does not require specialized experimental skill, and opens up new possibilities for routine or research applications. The main drawback of the GFP-based assays, however, is the use of HCMV MIE promoter/enhancer to

drive the GFP gene. Any antiviral agent that affects the expression of MIE promoter/enhancer would affect the GFP-based assay. As mentioned earlier, inhibition of MIE gene expression may not linearly correlate with inhibition of virus multiplication. The recombinant HCMV described in this report based on the luciferase gene driven by HCMV late pp28 promoter directly measure the effect of antiviral agents on virus replication. The luciferase-based assays can also be used not only for antiviral assays as shown above but also used for the study of HCMV gene expression. The advantages of using luciferase as a reporter include the simplicity of handling, reliability of quantification, and the possibilities of combining the system with confirmation tests as well as rapid procedure. In fact, luciferase assay using recombinant HCMV/pp28 requires only 4 days compared to the 14 days with the conventional plaque assays.

Acknowledgments

The authors wish to thank Dr. Gary S. Hayward (Johns Hopkins University, MD, USA) for providing recombinant virus HCMV/pp28-luc. This work was supported by a grant from the Molecular Medicine Research Fund by STEPI (98-J03-02-02-A-05).

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